Figure 16: Chromatography on Butyl-Sepharose 4 FF

Figure 17: Circular map of rhPBGD-His expression plasmid pExp2

Figure 18: PBGD reaction mechanism

Figure 19: DEAE chromatography elution profile

Figure 20: SDS-PAGE gel of DEAE eluates

Figure 21: Cobalt chromatography elution profile

Figure 22: SDS-PAGE gel results of cobalt eluates

Figure 23 and

Figure 24: Ilustrate numbers in diagrams (Table19). The expression of PBGD in HeLa cells was increased up to 475 times from the basal activity and in NIH 3T3 cells up to 11 times.

Figure 25: Comparison of fermentations PD05 and PD06 with strain PBGD-2

Figure 26: Comparison of fermentations PD09, PD11 and PD12

Figure 27: Comparison of fermentations PD09, PD11 and PD12 with strain PBGD-1.

Figure 28: Comparison of fermentations PD14, PD16 and PD19 with strain PBGD-2.

Figure 29: Comparison of fermentations PD14, PD16 and PD19 with strain PBGD-2

Figure 30: Comparison of fermentations PD19, PD21 and PD22 with strain PBGD-2.

Figure 31: Comparison of fermentations PD19, PD21 and PD22 with strain PBGD-2.

Figure 32: Comparison of fermentations PD19, PD1501 and PD1502

Figure 33:: Comparison of fermentations PD19, PD1501 and PD1502 with strain PBGD-2.

Figure 34:

Figure 35: Stability studies: Single use aliquots of extract were routinely taken out of the freezer (-20°C) and the rhPBGD-activity was measured and plotted over time.

Figure 36.Description of oligos used for PCR amplification.

Figure 37 A, and B:Strategy for PCR cloning of ALAD

Figure 38: Plasma levels of rhPBGD following administration to mice. 50 µg rhPBGD (2,3-2,8 mg/kg)

Figure 39: PBGD enzymatic activity in plasma following rhPBGD administration to mice

Figure 40: The urinary content of PBG and ALA in AIP-mouse treated with phenobarbital.

Figure. 41shows the urinary content of PBG and ALA in AIP-mouse treated with phenobarbital and rhPBGD.

Figure 42: Shows the grip strength analysis in control and AIP-mice

Figure 43: Rotarod analysis in control and AIP-mice. The rotarod analysis were determined using a rotarod treadmill (Ugo Basile) in wild type controls (control, n=5) and in AIP-transgenic mice (AIP, n=7).

Figure 44. Enzyme concentration over 8 weeks at 40°C measured by HPLC. A decrease from 2 mg/ml to 0,5 mg/ml and 8 mg/ml to 2,5 was detected.

Figure 46. Enzyme specific activity measured during 8 weeks at 40°C. The activity was measured using the enzyme activity assay and the protein concentration was measured using HPLC.

Figure 45. The enzyme activity measured over 8 weeks at 40°C. A significant decrease over the first week was seen for the high concentration sample, 1b. After two weeks the decrease rate was the same for all samples.

Figure 47. rhPBGD concentration over 12 weeks at –20°C (freezer), 5°C (fridge), 25°C (RT) and freeze/thawed at each sampling. The measurement was performed using HPLC

Figure 48. rhPBGD activity over 12 weeks at –20°C (freezer), 5°C (fridge), 25°C (RT) and freeze/thawed at each sampling.

Figure 49. rhPBGD specific activity over 12 weeks at –20°C (freezer), 5°C (fridge), 25°C (RT) and freeze/thawed at each sampling. Measurements were performed using enzyme activity assay and HPLC.

Figure 51. The rhPBGD activity measured over 8 weeks. The stability study has been performed under nitrogen at –20°C ± 5°C, 5°C ± 3°C and at 25°C ± 2°C.

Figure 51. The rhPBGD activity measured over 8 weeks. The stability study has been performed under nitrogen at –20°C ± 5°C, 5°C ± 3°C and at 25°C ± 2°C.

Figure 52. The specific rhPBGD activity measured using the enzyme activity assay and BCA protein concentration assay. The stability study has been performed under nitrogen at –20°C ± 5°C, 5°C ± 3°C and at 25°C ± 2°C.

Sequence list:

Seq. ID NO 1: Sequence of the expression plasmid pExp1-M2-BB

Seq. ID NO 2: Sequence of the EcoR I - Hind III linear fragment used for transformation in the hemC disruption strategy

Seq. ID NO 3: Sequence of the erythropoietic form (PBGD 1.1)

Seq. ID NO 4: Sequence of the non-erythropoietic form (PBGD 1.1.1)

Seq. ID NO 5: Sequence of PDGB from Spleen (PBGD 1.3)

Seq. ID NO 6: Sequence of PDGB from bone marrow (PBGD 2.1)

Seq. ID NO 7: Sequence of PDGB from bone marrow (PBGD 2.2)

Seq. ID NO 8: Sequence of PDGB from lymph node (PBGD 3.1)

Seq. ID NO 9: Sequence of PDGB from lymph node (PBGD 3.3)

7.A II. Development of lab scale fermentation

The overall strategy for the development of the fermentation process was outlined as follows. The use of a minimal medium in the inoculum steps should facilitate the stability of the host, and a minimal medium supplemented with yeast extract and peptone should facilitate growth and production in the main fermentation. To reach high cell densities a concentrated glucose feed was used to control the growth rate in the feed phase. In the expression plasmid pExp1-M2-BB the *rop* gene has been deleted [8] which means that the expression of rhPBGD can be temperature regulated. Initially it was decided to start with a fermentation temperature of 30 °C, which means that no temperature induction was used. If the productivity at this temperature was unsatisfactory the temperature could be increased to 37 °C or 42 °C to increase the productivity. Oxytetracycline was chosen as selection pressure, but if possible with regard to plasmid stability, the main fermentation should run without any selection pressure at all.

7.A.1 Initial batch experiments

The study was initiated 1/2/99 and the intermediary strain PBGD-1 was delivered 4/2/99. An initial M9H-Tc (6 mgl^{-1})(attachment 2, table 2) shake flask cultivation (PD03) was performed to study the growth in the M9H-Tc (6 mgl^{-1}) inoculum medium recommended by Icogen Inc. A fermenter medium designated MM5Y-Tc (6 mgl^{-1}) was designed based on BioGaia Fermentations know how from other recombinant *E.coli* fermentations. This medium was first tested in a shake flask cultivation (PD04) before two 1 L batch fermentations (PD05 and PD06) were performed with two variants of the medium. PD06 was performed in MM5Y-Tc (6 mgl^{-1}) substrate complemented with 2 gl^{-1} tryptone (table 8) to investigate if tryptone could facilitate growth .

In a batch cultivation exponential growth continues for a relatively few generations until nutrients are depleted or toxic products accumulates. Due to this growth begins to slow and thereafter the micro organisms enter the stationary phase, where a steady state cell number is reached.

In all these batch experiments the initial glucose concentration was 10 gl^{-1}. The parameters defined below were analysed or calculated, and the results are summarised in table 34 below.

Maximum growth rate (μ_{max}) $dX / dt = \mu \times X$, where X = Dry cell weight

7.A.3 III. Scale up of fermentation

The scale up part of the development was divided into two parts. First simulated large scale fermentations were performed in laboratory fermenters to study the effect of the increasing number of generations on plasmid stability and product quality. These tests were followed by the actual 850 L fermentations to investigate the effects from increasing the fermentation scale on plasmid stability and product quality.

BioGaia Fermentations 1500 L production fermenters are normally inoculated with broth from one 14 L fermenter. To mimic the fermentations PD14-PD19 the OD_{620} after inoculation should be approximately 0,1. When using a working volume of 9 L in the he 14 L fermenter the final OD_{620} necessary to achieve the same inoculum conditions in 850 L can be calculated as follows

$$OD_{620} \times 9\ L = 0{,}1 \times 850\ L \quad \rightarrow OD_{620} = 9{,}4$$

Inoculating the 14 L fermenter containing 9 L substrate with 500 ml broth (two 1 L shake flasks) with an OD_{620} of approximately 1,0 gives an OD_{620} initial of approximately 0,1-0,2. With a growth rate of 0,4 h^{-1} it will take 9-11 h to reach an OD_{620} of about 9. Since a growth rate of 0,4 h^{-1} is equivalent to a generation time of 1,7 h (ln2/0,4) this corresponds to 5-6 extra generations compared to the earlier lab scale fermentations.

An estimation of the number of generations in different steps of the entire process is given in table 11 below. The extra inoculum fermentation increases the total number of generations in the process with 15-25 %, an increase that could have effects on plasmid stability and product quality.

Table 37. Estimated number of generations in different process steps

Process step	Estimated number of generations
M9H-Tc agar plates	8 - 12
0,25 L M9H-Tc Shake flasks	4 - 5
9 L Inoculum fermentation	5 – 6
850 L Main fermentation	9 - 11
Σ Total process	26 - 34

Pressure Bar	Number of passages	Yield Total protein mg/ml	Yield Activity U/ml	Specific activity U/mg protein
800	1	1,2	4	3,3
600	3	1,6	7	4,3
800	3	3,5	16	4,6
1000	3	1,7	10	5,9

Exchanging the micro filter (0,2 µm) for ultra filters (500 K and 1000 K) resulted in less fouling at the filter surface and protein concentration increased to acceptable yields (Table 16). The smaller membrane area and flatter surface minimised product hold up and adsorption, which in this case increases yields. Furthermore, the transmission of total protein seems to be lower than the transmission of rhPBGD when using an ultra filter (1000 K), resulting in a higher specific activity (table 16). In all experiments the homogenate was dia filtered to the same theoretical yield (90%) to enable comparison of the results.

Final statement: For scaling up to production scale a 1000 K filter was chosen for cell debris removal since this filter gave a good yield of rhPBGD with a high specific activity. Diluted homogenate was decided to be concentrated approximately 2,5 times and then dia filtered with 50 mM sodium-phosphate, 1,34 mM EDTA, pH 7,4 to get a theoretical yield of rhPBGD of about 95 %. Nitrogen was flushed over the permeate surface in order to prevent oxidation (PD1502). Permeate-flux was set at 15 l $m^{-2}h^{-1}$ and the temperature was set at 15- 25°C (PD1501-PD1502).

Table 42. Summary of rhPBGD yield from Batch PD21 using different filters during cell debris removal. Homogenisation parameters: 800 bar, 3 passages. The yield of rhPBGD from membrane filtration was compared to centrifuged material.

Cell debris removal by	Yield Total protein mg/ml	%*	Yield Activity U/ml	%*	Specific activity **U/mg protein**
0,2 µm filter	2,2	30	7	21	3,3
500 K filter	4,8	65	22	69	4,6
1000 K filter	3,1	42	21	66	6,8
centrifuged	–	80	–	90	5,1

* Yield is given in % compared to homogenate.

7.A.4.4 Final filtration

Membrane filtered extracts contained less particles and was thereby easier to filter than centrifuged extract, where problems with clogging on the filter surface occurred. The clogging made it difficult to perform integrity tests. A white slippery precipitate was always seen in extract before the final filtration. When analysing the dissolved precipitate spectrofotometrically at OD_{260} /OD_{280} resulting in a ratio near 2, it was concluded that it contained nucleic acid.

7.A.4.5 Scale up of down stream process (PD22, PD1501 and PD1502)

The entire final process was at first tested at a 15 L scale (batch PD22) ending with a rhPBGD yield of 75 %. When scaling up to 300 L(PD1501) problems with precipitation in the broth occurred and the rhPBGD yield decreased to 46 %. When processing batch PD1502 no precipitation was seen and the rhPBGD yield increased to 77 %. The results are summarised in tables 43 and 44.

The low yields of rhPBGD from batch PD1501 was probably due to several factors: Using the same filter unit at cell concentration and cell debris removal commonly saves both money and time but when a white precipitate occurred in the broth it resulted in problems cleaning the filter between cell concentration and cell debris removal. The composition of the white insoluble precipitate was analysed and the results are shown in table 19. To avoid the precipitate formation in PD1502 the substrate preparation was carefully monitored. No new component was added until the former component was completely dissolved. No precipitate was formed in batch PD1502.
The filter area was small in comparison to the processed volume, which increased the chances for clogging on the filter surface. Dia-filtration was only performed to achieve a theoretical yield, rhPBGD, of 90 %.

In batch PD1502 the concentration of rhPBGD in the extract was low compared to batch PD22 but the yield was slightly higher. The lower concentration was due to an operator mistake using an increased dia filtration volume at cell debris removal during the process of PD1502. If a smaller dia filtration volume during cell debris removal in batch PD1502 had been used it would have resulted in a higher concentration of rhPBGD but the yield had then decreased.

at 72°C was used at the end to ensure that the extension products were filled out. One of this PCR mix was again amplified exactly as described above and cloned into pBluescript II SK- (Stratagene, catalogue # 212206), linearized with *Eco*R I and *Hind* III after purification (using GEANECLEAN III, from BIO 101 catalogue # 1001-600) and digestion with the same two enzymes (see Figure 37, A and B).

8.2 Sequencing

Four plasmid clones from the above ligation viz. pBlueAlaD-1-4 were sequenced with the Big Dye terminator cycle sequencing kit from PE/ABI catalogue # 4303152. Three vector primers, ICO383 (5' GTAATACGACTCACTATA GGGC 3'), ICO384 (5' CTAAAGGGAACAAAAGCTGGAG 3') and IC0618 (5- GCGCGTAATACGACTCACTA 3) and two ALAD-specific primers, ICO616 (5' CCTACGCTGTGTCTTGATCT 3') and ICO617 (5' GGCTT CACCATGAGCATGTC 3') were used. The results are tabulated in Table 46.

Table 46 Summary table of sequencing results

Clone #	Nucleotide change	Amino acid Change
BlueAlaD-1	-	-
BlueAlaD-2	-	-
PBlueAlaD-3	168, T to C 414, C to T 463, T to C 866, C to T	56, Y (silent) 138, N (silent) 155, L (silent) 289, A to V
PBlueAlaD-4	180, T to C	56, y (silent)

Reporting and Results

The inserts in all four clones confirmed to be ALAD by sequence analysis. The results are shown in Table 1. As seen, two of the clones completely match the published sequence (2). The other two have changes, most of which are silent. Without a larger sampling volume it is difficult to distinguish between allelic variation and PCR/cloning artifacts. The

ALAD insert from pBlueAlaD-2 was used for expression purposes and its sequence is shown in seq. 14.

Evaluation and conclusions

The PCR amplification strategy used has generated ALAD cDNA that matches the published sequence. It has convenient restriction sites at the ends for ease of manipulation into expression vectors, including an engineered BsrD I site just upstream of the ATG.

Example 9

Administration for rhPBGD, an animal study.

Recombinant human Porphobilinogen Deaminase (rhPBGD) will be administered as an enzyme substitution treatment for patients diagnosed with Acute Intermittent Porphyria (AIP). rhPBGD will be administered by s.c or i.v injections. It is essential for the efficacy of the treatment, e.g. reduction of the toxic precursors porphobilinogen (PBG) and δ-aminolevulinic acid (ALA), that rhPBGD can enter the blood stream and remain biologically active.

Pharmacokinetics of rhPBGD

To study the pharmacokinetics, wildtype healthy B6 mice were injected with rhPBGD. The content and enzyme activity of rhPBGD was followed in plasma from animals after different timepoints (0, 15, 30, 45 and 60 min). Each animal received one single injection of 50 µg rhPBGD and three different routes of administration were used, i.v, i.p or s.c. The plasma levels of rhPBGD analysed by ELISA are shown in Figure 1. The conclusion from this data is that the half-life of rhPBGD following i.v injection is 20-30 min. Following i.p injection the maximal levels of rhPBGD was found after approximately 30 min. Also s.c injections of rhPBGD resulted in detectable levels of PBGD in plasma which shows that it is possible to use this route for administration. S.c injection did also result in a slow release of rhPBGD to plasma with maximal levels found in the last timepoint analysed (60 min).

38 shows plasma levels of rhPBGD following administration to mice. 50 µg rhPBGD (2,3-2,8 mg/kg) were injected i.v, i.p or s.c to wildtype B6 mice. After different timepoints (0, 15,

In this study all animals were treated with an increasing dose of phenobarbital for four days (day 0-4, 75-90 mg/kg and day i.p). One group of animals did at the same time also receive rhPBGD for seven days (day 0-7, 1,7-2,3 mg/kg and day i.p). The content of PBG and ALA were analysed in 24 h urine samples where levels are expressed as mmol/mol creatinine. As seen in Figure 4 the rhPBGD treatment results in a lowering of urinary content of PBG and ALA as compared to animals treated with only phenobarbital (Figure 40). This data shows that rhPBGD, when given to mice with high levels of PBG and ALA in serum (acute AIP attack), can lower these levels as analysed by the urine content of this metabolites. No antibody formation against rhPBGD was seen in these animals when analysing at day 11 or at 2 weeks after that the rhPBGD treatment was stopped.

The conclusion from this data is that rhPBGD can lower the levels of PBG and ALA in mice during an acute attack of porphyria. This may also indicate that clinical symptoms seen in AIP patients, which probably are induced by the high serum levels of PBG and ALA, may be reversed by this treatment. Further studies are now on its way to confirm this data. It is also possible to increase the treatment period using rhPBGD in mice due to that no antibody formation was seen.

Fig 40 shows the urinary content of PBG and ALA in AIP-mouse treated with phenobarbital. Mice were treated with an increasing dose of phenobarbital for 4 days (day 0-4, 75-90 mg/kg and day i.p). PBG and ALA levels were analysed in 24-h urine samples and expressed as mmol/mol creatinine. Data from one representative animal are shown.

Fig. 41shows the urinary content of PBG and ALA in AIP-mouse treated with phenobarbital and rhPBGD. Mice were treated with an increasing dose of phenobarbital for 4 days (day 0-4, 75-90 mg/kg and day i.p) and rhPBGD for 7 days (day 0-7, 1,7-2,3 mg/kg and day i.p). PBG and ALA levels were analysed in 24-h urine samples and expressed as mmol/mol creatinine. Data from one representative animal are shown.

Ongoing studies in mice

Known clinical symptoms in AIP patient are different neurological symptoms such as pain in stomach and/or legs and arms and muscle weakness. To study these symptoms in the transgenic mice we also analysed the motorneuron function by different behavioural tests such as rotarod and grip strength. Data shows that the transgenic AIP-mouse have

significantly lower activity in all behavioural tests as compared to wildtype controls. See examples of such data from the grip strength (Figure 5) and from rotarod (Figure 6). Motor neuropathy has also been described in the AIP-mice by Lindberg, R. L. P. et al. Journal of Clinical Investigation 103:1127-1134, 1999. We will now analyse if also the neurological disorders in the AIP-mouse can be can be reversed by the rhPBGD treatment, as a possible long-term treatment for patients.

Fig 42 shows the grip strength analysis in control and AIP-mice. Grip strength were determined using a grip strength meter (Ugo Basile) in heterozygous control animals (control 1, n=5), in wild type controls (control 2, n=5) and in AIP-transgenic mice (AIP, n=5).

Fig. 46 shows a rotarod analysis in control and AIP-mice. The rotarod analysis were determined using a rotarod treadmill (Ugo Basile) in wild type controls (control, n=5) and in AIP-transgenic mice (AIP, n=7).

Example10

Stability data for rhPBGD

Stability study 1 - Selection of formulation buffer

To find the best suitable formulation conditions for rhPBGD-His, the enzyme was formulated in a phosphate buffer containing mannitol and glycine as protein stabilizers. Different pH, ion strength and enzyme concentration were investigated, Table 47. The study was performed at 40°C and 75% relative humidity during 8 weeks.

Table 47. Formulations

Sample no.	pH	Ion strength (mM)	rhPBGD-His conc. (mg/ml)
1a, 2c, 3a	7,5	10	2
1b	7,5	10	8
2a	6,5	10	2
2b	7,0	10	2
2d	8,0	10	2

2e	8,5	10	2
3b	7,5	50	2
3c	7,5	100	2

The samples were sterile filtered and aliquots of 170 l were dispensed into 300 l glass vials with a Teflon/silicon crimp cap. Samples were collected for analysis on week 0, 1, 2, 4 and 8 and were analysed for enzyme activity (enzyme activity assay), protein concentration (HPLC) and degradation/aggregation (HPLC, SDS-PAGE and IEF).

The enzyme precipitated in all samples except for sample no. 2e (pH 8,5). Sample no. 1b (8 mg/ml) showed a higher precipitation rate than samples with lower enzyme concentration. This sample turned yellow after one week, all other samples turned yellow after 2 weeks. The HPLC chromatograms showed that a prepeak to the rhPBGD-His peak was formed during storage at 40°C, and that this prepeak increase with time. The peak for pure rhPBGD-His decreased with time. Figure 1, shows the amount enzyme calculated from the area under the peak in HPLC chromatograms.

The enzyme activity, Figure 45, decreased from 16 Units/ml (U/ml) to 2 U/ml in 8 weeks, for sample no. 1b the decrease in enzyme activity was more pronounced the first week, 40 U/ml to 10 U/ml, corresponding to the precipitation seen in Figure 44. The specific enzyme activity is shown in Figure 3. It seemed like high protein concentration was detrimental for the enzyme activity (sample no. 1b).

SDS-PAGE gels showed bands from aggregates as well as from scissoring after one week for all samples.

On the isoelectric focusing, IEF, gels four bands corresponding to the four catalytic forms of the enzyme (E, E1, E2 and E3) were seen on day 0. During storage at 40°C it seemed like the first band (E) was weakened and the second band (E1), which is more acidic was getting stronger. The number of more acidic bands increased over time. This was probably due to deamidation.

Conclusion

rhPBGD-His was not stable at 40°C. However, the only formulation buffer in which no visible precipitation of the enzyme was detected was no. 2e (pH 8,5). High concentration of

CLAIMS

1. A method for treatment or prophylaxis of disease caused by deficiency, in a subject, of one or more enzymes belonging to the heme biosynthetic pathway, the method comprising administering, to the subject, an effective amount of one or more catalysts which is/are said enzyme(s), or an enzymatically equivalent part or analogue thereof,
optionally in combination with gene therapy of a mutation related to the a desease of the heme biosynthetic pathway

2. A method according to claim 1, wherein the disease is selected from the group consisting of
acute intermittent porphyria (AIP),
ALA deficiency porphyria (ADP),
Porphyria cutanea tarda (PCT),
Hereditary coproporphyria (HCP),
Harderoporphyria (HDP),
Variegata porphyria (VP),
Congenital erythropoetic porphyria (CEP),
Erythropoietic protoporphyria (EPP), and
Hepatoerythropoietic porphyria (HEP).

3. A method according to claim 1, wherein the catalyst is one or more enzymes selected from the group consisting of
delta-aminolevulininic acid synthetase,
delta-aminolevulinic acid dehydratase (ALAD),
porphobilinogen deaminase (PBGD),
uroporphyrinogen III cosythetase,
uroporphyrinogen decarboxylase,
coproporphyrinogen oxidase,
protoporphyrinogen oxidase, and
ferrochelatase,
or an enzymatically equivalent part or analogue thereof.

4. A method according to claim 1, wherein the disease is AIP and the enzyme is PBGD or an enzymatically equivalent part or analogue thereof, preferable in combination with ALAD

5. A method according to claim 1, wherein the catalyst is a recombinant form of the enzyme belonging to the heme biosynthetic pathway or of the enzymatically equivalent part or analogue thereof.

6. A method according to claims 1, wherein the catalyst is administered by a route selected from the group consisting of the intravenous route, the intraarterial route, the intracutaneous route, the subcutaneous route, the oral route, the buccal route, the intramuscular route, the anal route, the transdermic route, the intradermal route, and the intratechal route.

7. A method according to any of claim 1, wherein the catalyst is formulated in an isotonic solution, such as 0.9% NaCl and 10-50 mM Sodium phosphate pH 6.50 to 8 or Sodium phosphate, glycine, mannitol or the corresponding potassium salts.

8. A method according to any of claim 7, wherein the catalyst is lyophilised.

9. A method according to any of claim 8, wherein the catalyst is sterile filtered.

10. A method according to any of claim 1, wherein the catalyst is formulated as lipid vesicles comprising phosphatidylcholine or phosphatidylethanolamine or combinations thereof.

11. A method according to any of claim 1, wherein the catalyst is incorporated into erythrocyte ghosts.

12. A method according to any of claim 1, wherein the catalyst is formulated as a sustained release formulation involving biodegradable microspheres, such as microspheres comprising polylactic acid, polyglycolic acid or mixtures of these.

13. A method according to any of claim 1, wherein the catalyst is lyophilized in a two-compartment cartridge, where the catalyst will be in the front compartment and water for reconstitution in the rear compartment.

14. A method according to claim 13, wherein the two compartment cartridge is combined with an injection device to administer the catalyst either by a needle or by a needle-less (high pressure) device.

15. A method according to any of claim 1, wherein the catalyst is formulated in a physiological buffer containing an enhancer for nasal administration.

16. A method according to claim 1, wherein the catalyst is formulated as an oral formulation containing lipid vesicles, such as those comprising phospatidylcholine, phosphatidylethanolamine, or sphingomyeline, or dextrane microspheres.

17. A method according to claim 1, wherein the catalyst is formulated so as to enhance the half-life thereof in the subject's bloodstream.

18. A method according to claim 17, wherein the catalyst has a polyethylene glycol coating.

19. A method according to claim 17, wherein the catalyst is complexed with a heavy metal.

20. A method according to claim 1, wherein the catalyst is an enzymatically equivalent part or analogue of the enzyme and exerts at least part of its enzymatic activity intracellularly upon administration to the subject.

21. A method according to claim 20, wherein the catalyst is a small artificial enzyme or an organic catalyst which can polymerize porphobilinogen to hydroxymethylbilane

22. A method according to claim 1, wherein the catalyst is said enzyme formulated in such a manner that it exerts at least part of its enzymatic activity intracellularly upon administration to the subject.

23. A method according to claim 22, wherein the catalyst is tagged with specific carbohydrates or other liver cell specific structures for specific liver uptake.

24. A method according to claim 1, wherein the catalyst exerts substantially all its enzymatic activity extracellularly in the bloodstream.

25. A method according to claim 24, wherein the enzymatic activity of the catalyst on its relevant heme precursor results in a metabolic product which 1) either moves into the intracellular compartment and is converted further via the remaining steps of the heme biosynthetic pathway or 2) is excreted from the subject via urine and/or faeces.

26. A method according to claim 1, wherein the catalyst has been prepared by a method comprising

a) introducing, into a suitable vector, a nucleic acid fragment which includes a nucleic acid sequence encoding the catalyst;
b) transforming a compatible host cell with the vector;
c) culturing the transformed host cell under conditions facilitating expression of the nucleic acid sequence; and
d) recovering the expression product from the culture
and optionally subjecting the expression product to post-translational processing, such as in vitro protein refolding, enzymatic removal of fusion partners, alkylation of amino acid residues, and deglycosylation, so as to obtain the catalyst.

27. A method according to claim 1, wherein the catalyst has been prepared by liquid-phase or solid-phase peptide synthesis.

28. A method according to claim 26 of the preceding claims, wherein the catalyst is free from any other biological material of human origin.

29. A method according to any claim 1, wherein the catalyst is administered at least once a day, such as 2, 3, 4, or 5 times daily.

30. A method according to claim 29 wherein the daily dosage is in the range of 0.01 – 1.0 mg/kg body weight per day, such as in the range of 0.05 – 0.5 mg/kg body weight per day.

31. A method according to claim 29, wherein the daily dosage is about 0.1 mg per kg body weight per day.

32. A method according to claims 1 wherein the catalyst is a recombinant form of the enzyme.

33. A method according to claim 32 wherein the catalyst is recombinant human PBGD based on any of Seq. ID NO 3 (clone PBGD 1.1) and Seq. ID NO 4 (non-erythro PBGD 1.1.1).

34. A method for treating a patient having a mutation in the PBGD gene causing an enzyme defect, comprising the use of a human PBGD cDNA sequence of either non-erythropoietic form or erythropoietic form according to whether the tissue in which PBGD should be expressed is in cells of erythroid origin or in other cells, and transfection of the patient with the relevant cDNA.

35. The method according to claim 34, wherein the enzyme defiency is selected from enzyme defiencies resulting in a disease selected from Acute Intermittent Porphyria, (AIP), ALA deficiency porphyria (ADP), Porphyria cutanea tarda (PCT), Hereditary coproporphyria (HCP), Harderoporphyria (HDP), Variegata porphyria (VP), Congenital erythropoietic porphyria (CEP), Erythropoietic protoporphyria (EPP), and Hepatoerythropoietic porphyria (HEP).

36. The method according to claim 35 wherein the disease is Acute Intermittent Porphyria, (AIP).

37. The method according to claim 34, wherein the human PBGD cDNA sequence is selected from Seq. ID NO 3 (clone PBGD 1.1) and Seq. ID NO 4 (non-erythro PBGD 1.1.1).

38. The method according to any of claims 34 wherein the transfection is by use of a vector selected from adenovirus, retrovirus and associated adenovirus.

39. The method according to claim 34 wherein the PBGD transfection of the patient (erythropoeitic and/or non-erythropoietic cells) results in substantially normal PBGD activity measured as a normalisation in urinary and/or serum levels of delta-aminolevulinic acid (ALA) and porphobilonogen (PBG) compared to the levels before treatment or to a reduction in the frequency of attack of symptons.

40. A method of gene therapy treatment of patients with Acute Intermittent Porphyria (AIP) by a correction of one of the specific point mutations identified causing AIP by use of chimeraplasty gene repair.

41. The method according to claim 40 comprising a delivery system for transfection which is by use of non-viral vectors formulated in a vehicle preparation comprisng one or more components selected from cationic phospholipids, phospholipids, phospholipids mixed with neutral lipids, lictosylated PEI, liposomes liposomes comprising mixtures of natural phopholipids and neutral lipids.

42. A method according to claim 40 wherein the mutation is selected from Table A.

43. A catalyst which is an enzyme of the heme biosynthetic pathway or an enzymatically equivalent part or analogue thereof, for use as a medicament.

44. A catalyst according to claim 43, which is recombinant human PBGD based on any of Seq. ID NO 3 (clone PBGD 1.1) and Seq. ID NO 4 (non-erythro PBGD 1.1.1).

45. An expression plasmid pExp1-M2-BB as shown in Seq. ID NO 1 for use in the expression of rhPBGD in *E. coli.*

46. A DNA fragmet, EcoR I - Hind III linear fragment as shown in Seq. ID NO 2, capable of obtaining *hemC*-deletion in a host.

47. A production strain of rhPBGD obtained by use of the DNA fragmet, EcoR I - Hind III linear fragment as shown in Seq. ID NO 2 to obtain *hemC*-deletion in the host JM105-H-R6-C by homologous gene replacement and transforming the resulting strain with the expression plasmid pExp1-M2-BB to yield the final production strain PBGD which is free from production of PBGD of non human origin (Accsession No 12915).

48. A method for the preparation of rhPBGD by a method comprising

a) introducing, into a suitable vector, a nucleic acid fragment which includes a nucleic acid sequence encoding PBGD;

b) transforming a compatible host cell with the vector;

c) culturing the transformed host cell under conditions facilitating expression of the nucleic acid sequence;

d) recovering the expression product from the culture.

49. A method according to claim 48 further comprising a fermentation step.

50. A method according to claim 48 further comprising a purification step.

51. A method according to claim 50 wherein the purification is performed wiht a His-Tag (rhPBGD-His).

52. A method according to any of claims 1-52 wherein the patient is treated subcutaneusly with the catalyst.

53. 43. A catalyst which is an enzyme of the heme biosynthetic pathway or an enzymatically equivalent part or analogue thereof, for use as a medicament.

54. Use of one or more catalyst according to any of the preceding claims for the preparation of a medicament for treating a disease related to a disease in the heme biosynsthetic pathway.

55. Use according to claim 54 wherein the disease is is selected from the group comprising of

acute intermittent porphyria (AIP),

ALA deficiency porphyria (ADP),

Porphyria cutanea tarda (PCT),

Hereditary coproporphyria (HCP),

Harderoporphyria (HDP),

Variegata porphyria (VP),

Congenital erythropoetic porphyria (CEP),

Erythropoietic protoporphyria (EPP), and

Hepatoerythropoietic porphyria (HEP).

56. A rhPBGD having a stability of at least 6 weeks at -20°c, such as for at least 7 weeks, preferable for 8 weeks.

57. A rhPBGD having a stability resulting in a decrease in activity of less that 10% per month, such as less that 5%.

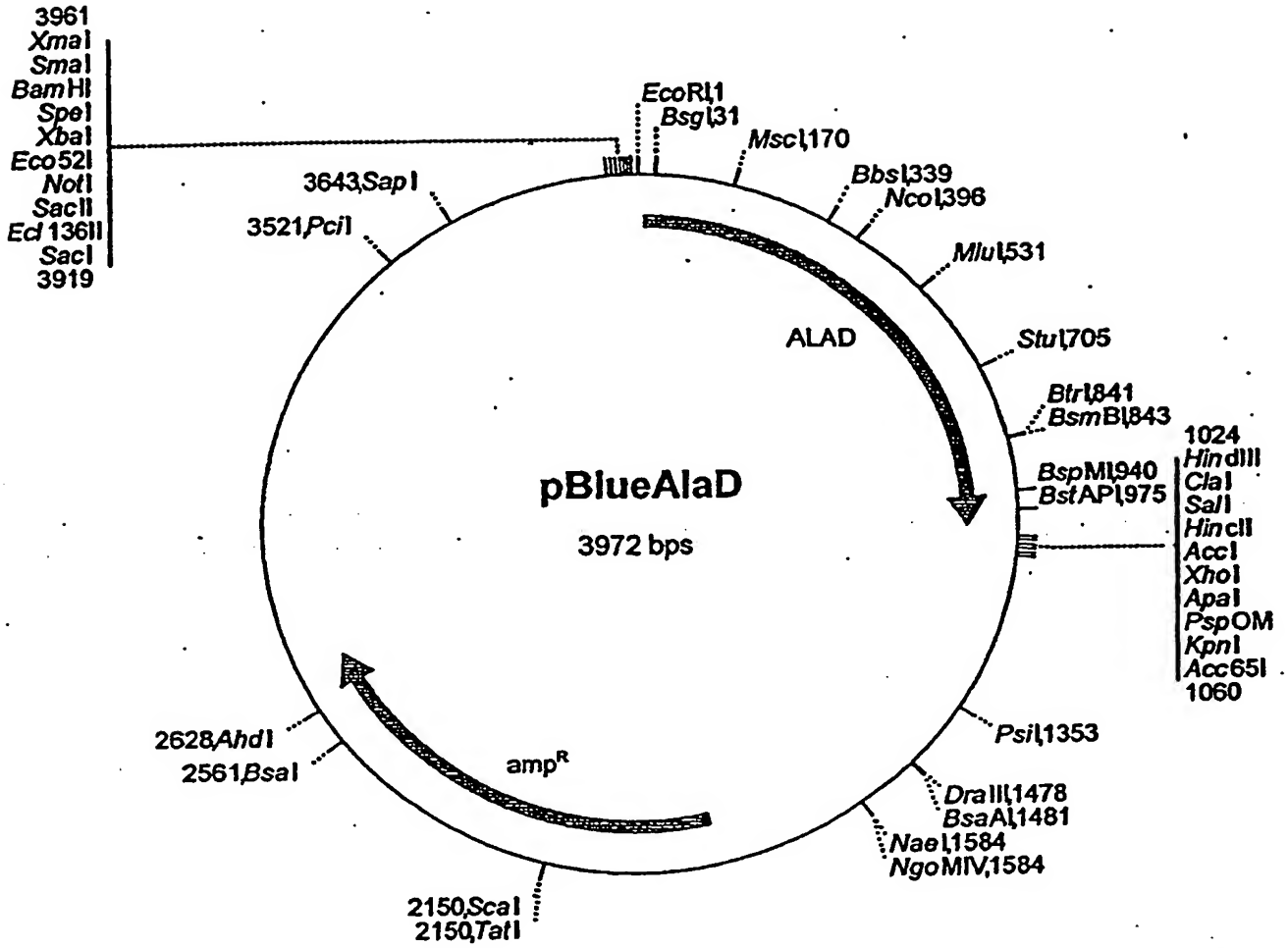
pBlueAlaD
3972 bps
ALAD
amp^R
3961
XmaI
SmaI
BamHI
SpeI
XbaI
Eco52I
NotI
SacII
Ecl136II
SacI
3919
EcoRI,1
BsgI,31
MscI,170
BbsI,339
NcoI,396
MluI,531
StuI,705
BtrI,841
BsmBI,843
1024
HindIII
ClaI
SalI
HincII
AccI
XhoI
ApaI
PspOM
KpnI
Acc65I
1060
BspMI,940
BstAPI,975
PsiI,1353
DraIII,1478
BsaAI,1481
NaeI,1584
NgoMIV,1584
2150,ScaI
2150,TatI
2628,AhdI
2561,BsaI
3643,SapI
3521,PciI

Fig. 38

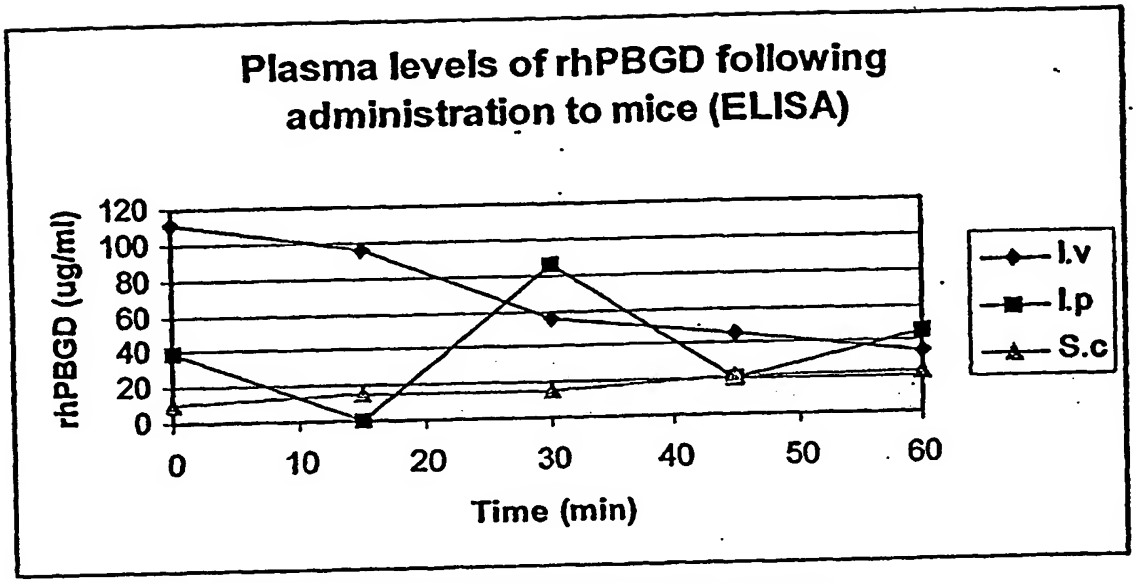

Figure 38: Plasma levels of rhPBGD following administration to mice.

Fig. 39

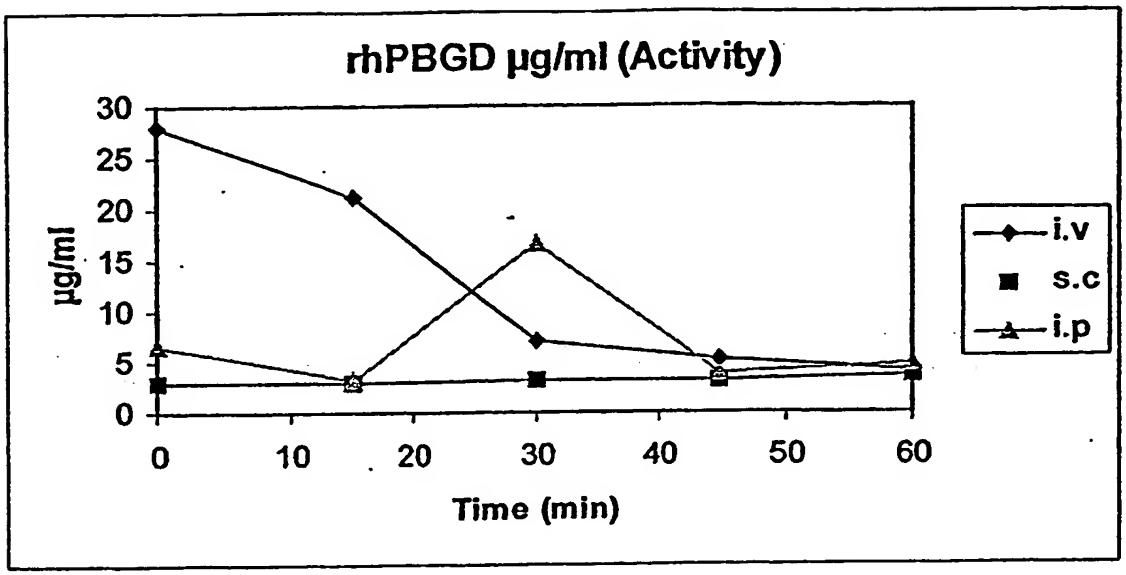

Figure 39: PBGD enzymatic activity in plasma following rhPBGD administration to mice

Fig. 40

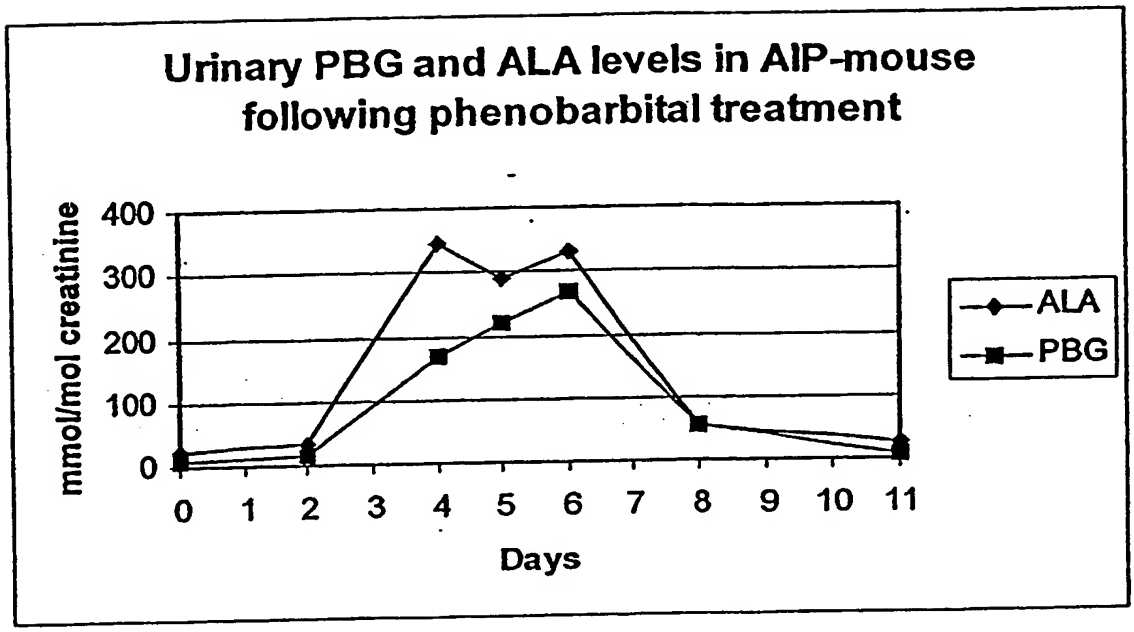

Figure 40: Urinary content of PBG and ALA in AIP-mouse treated with phenobarbital.

Fig. 41

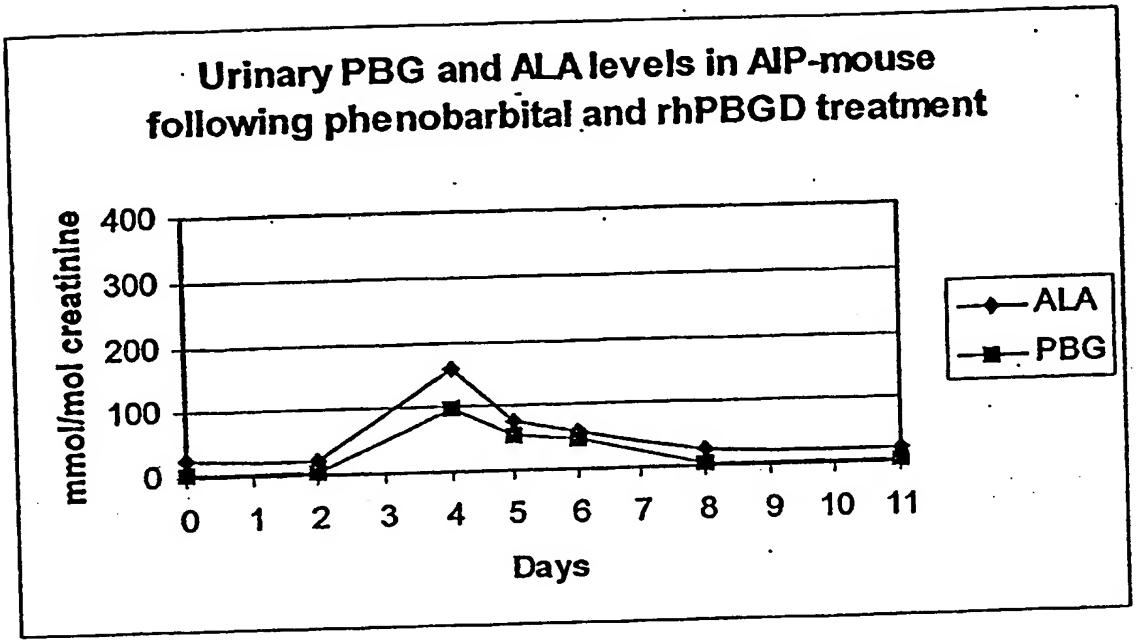

Figure 41: Urinary content of PBG and ALA in AIP-mouse treated with phenobarbital and rhPBGD. Mice were treated with an increasing dose of phenobarbital for 4 days (day 0-4,

Fig. 42

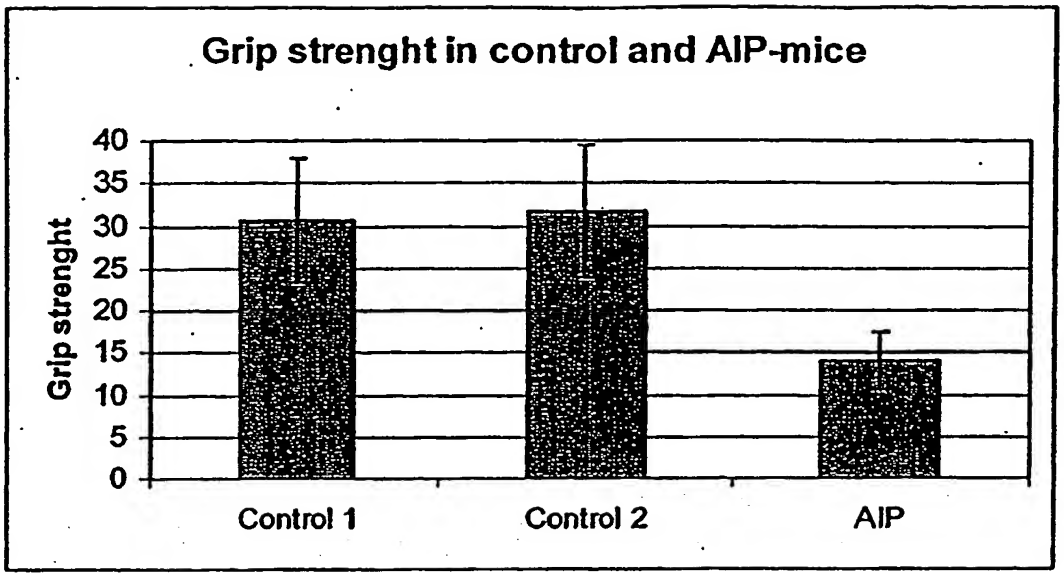

Figure 42: Grip strength analysis in control and AIP-mice. Grip strength were determined using a grip strength meter (Ugo Basile) in heterozygous control animals (control 1, n=5), in wild type controls (control 2, n=5) and in AIP-transgenic mice (AIP, n=5).

Fig. 43

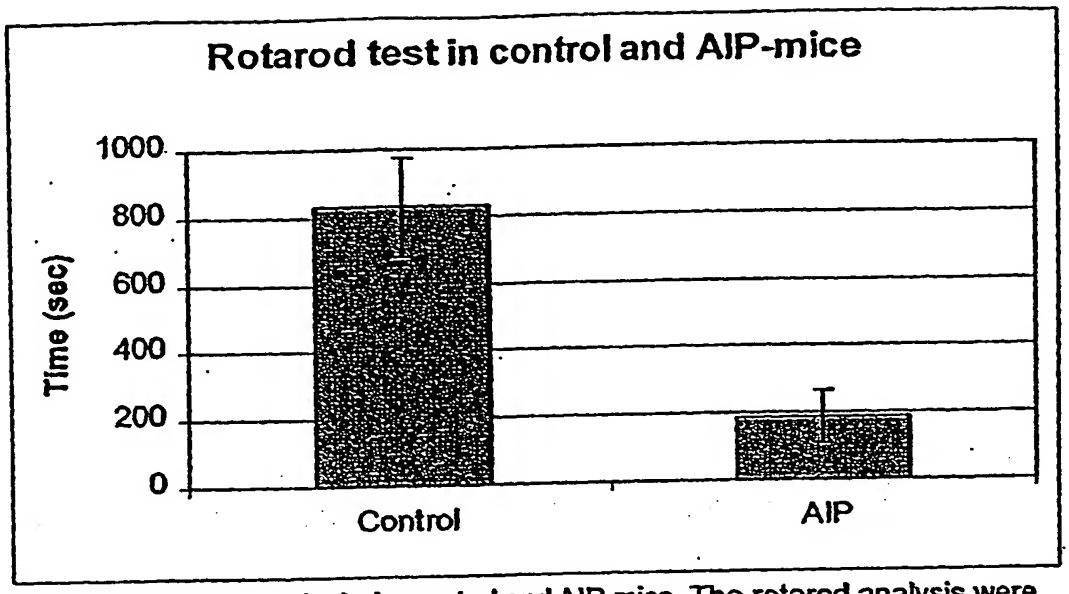

Figure 44 Rotarod analysis in control and AIP-mice. The rotarod analysis were determined using a rotarod treadmill (Ugo Basile) in wild type controls (control, n=5) and in AIP-transgenic mice (AIP, n=7).

Fig. 44

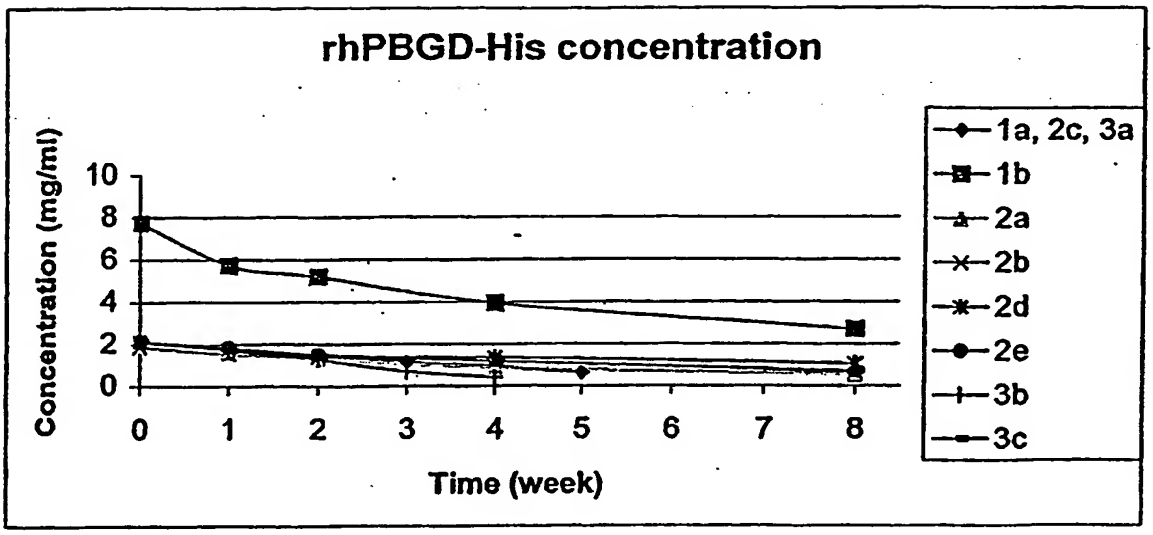

Figure 44. Enzyme concentration over 8 weeks at 40°C measured by HPLC. A decrease from 2 mg/ml to 0,5 mg/ml and 8 mg/ml to 2,5 was detected.

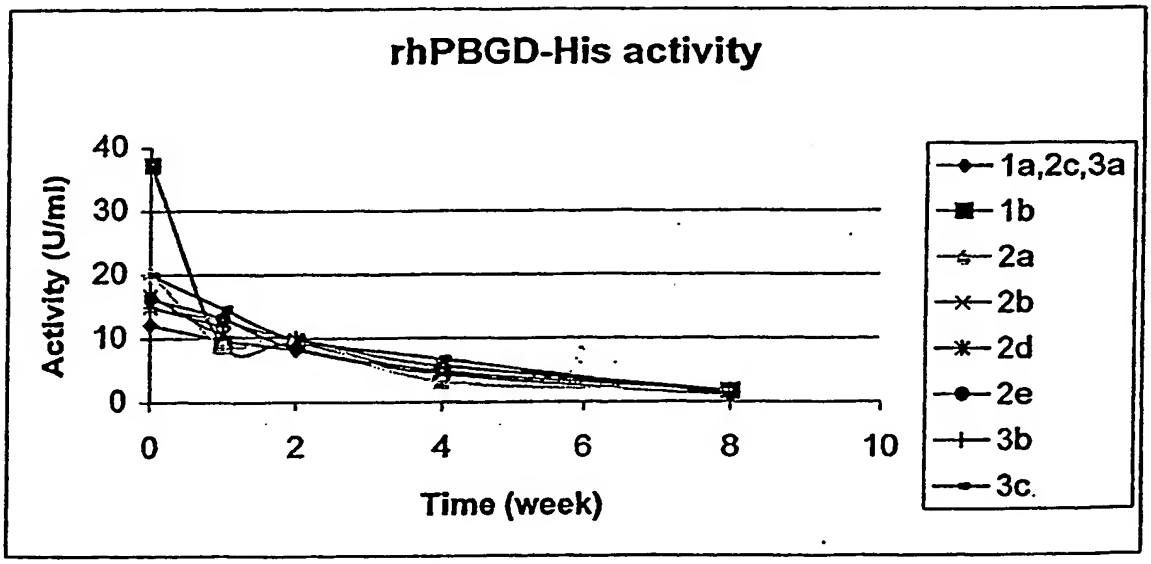

Figure 45. The enzyme activity measured over 8 weeks at 40°C. A significant decrease over the first week was seen for the high concentration sample, 1b. After two weeks the decrease rate was the same for all samples.

Fig. 45

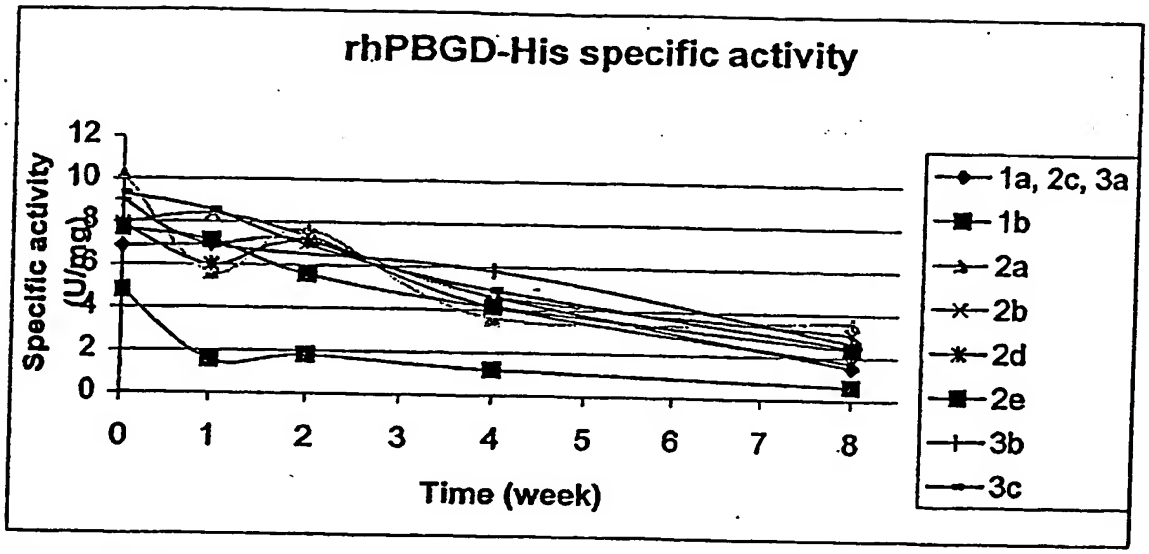

Figure 46. Enzyme specific activity measured during 8 weeks at 40°C. The activity was measured using the enzyme activity assay and the protein concentration was measured using HPLC.

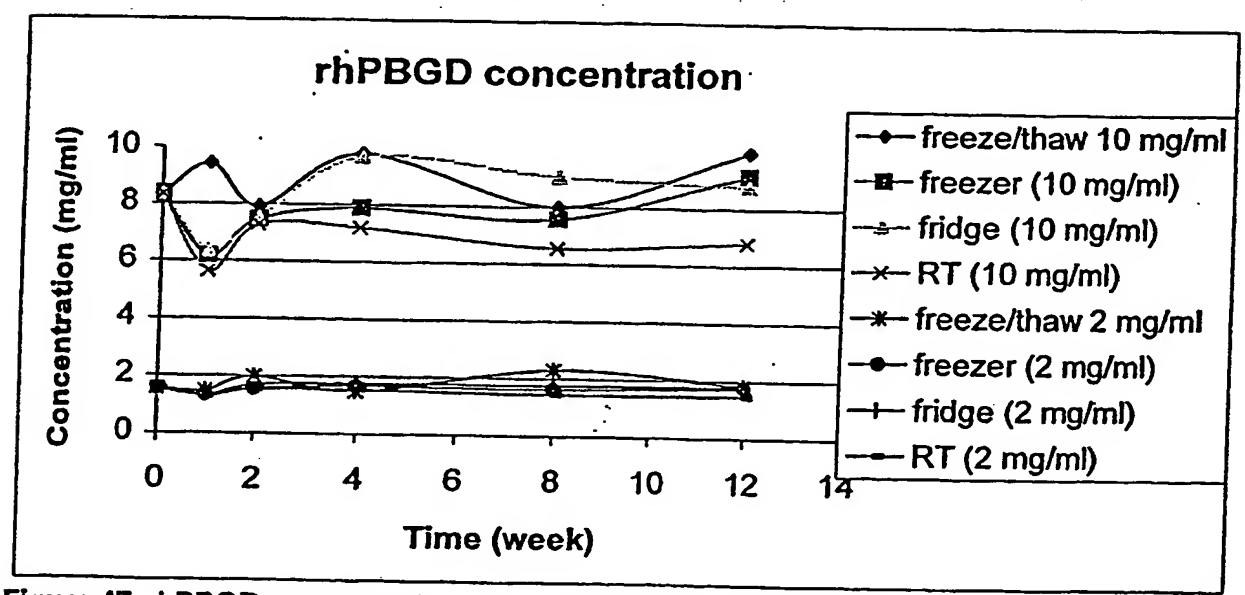

Figure 47. rhPBGD concentration over 12 weeks at –20°C (freezer), 5°C (fridge), 25°C (RT) and freeze/thawed at each sampling. The measurement was performed using HPLC.

Fig. 46

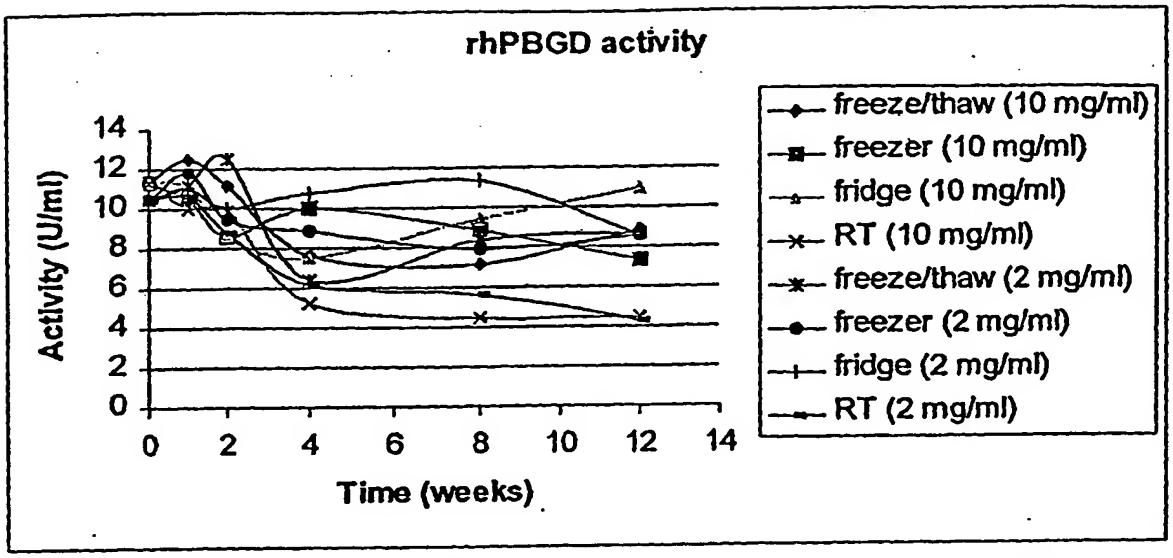

Figure 48. rhPBGD activity over 12 weeks at –20°C (freezer), 5°C (fridge), 25°C (RT) and freeze/thawed at each sampling.

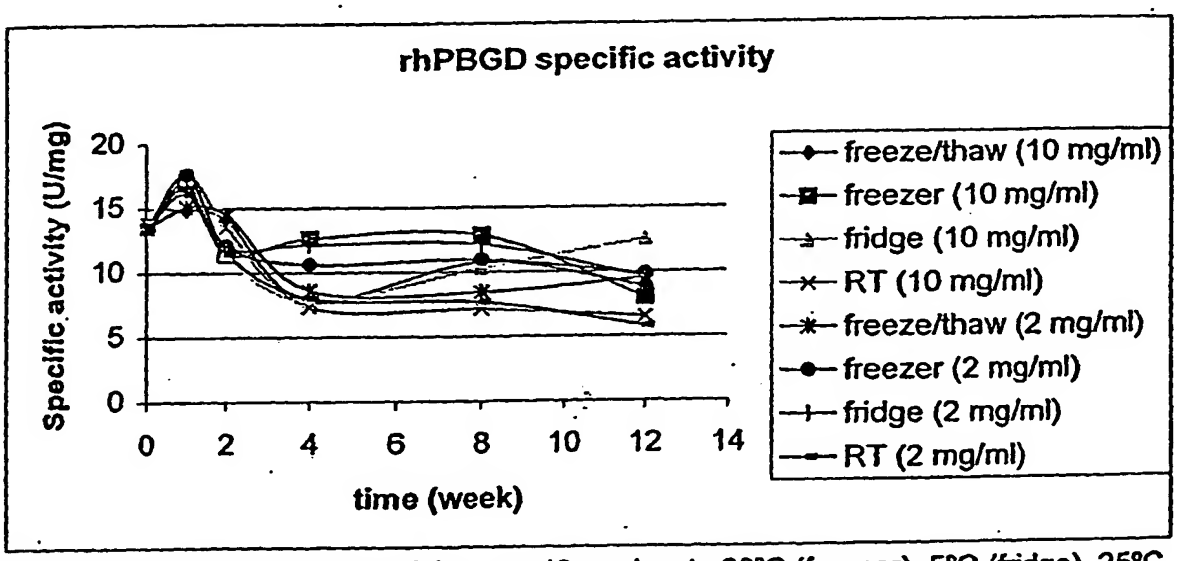

Figure 49. rhPBGD specific activity over 12 weeks at –20°C (freezer), 5°C (fridge), 25°C (RT) and freeze/thawed at each sampling. Measurements were performed using enzyme activity assay and HPLC.

Fig. 47

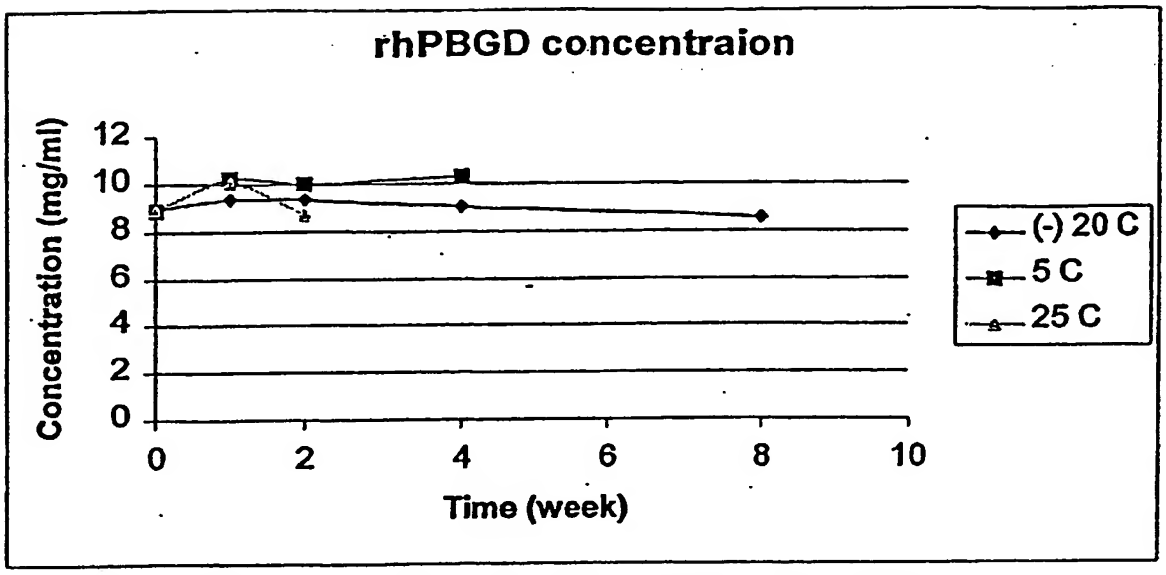

Figure 50. rhPBGD concentration measured over 8 weeks using BCA.

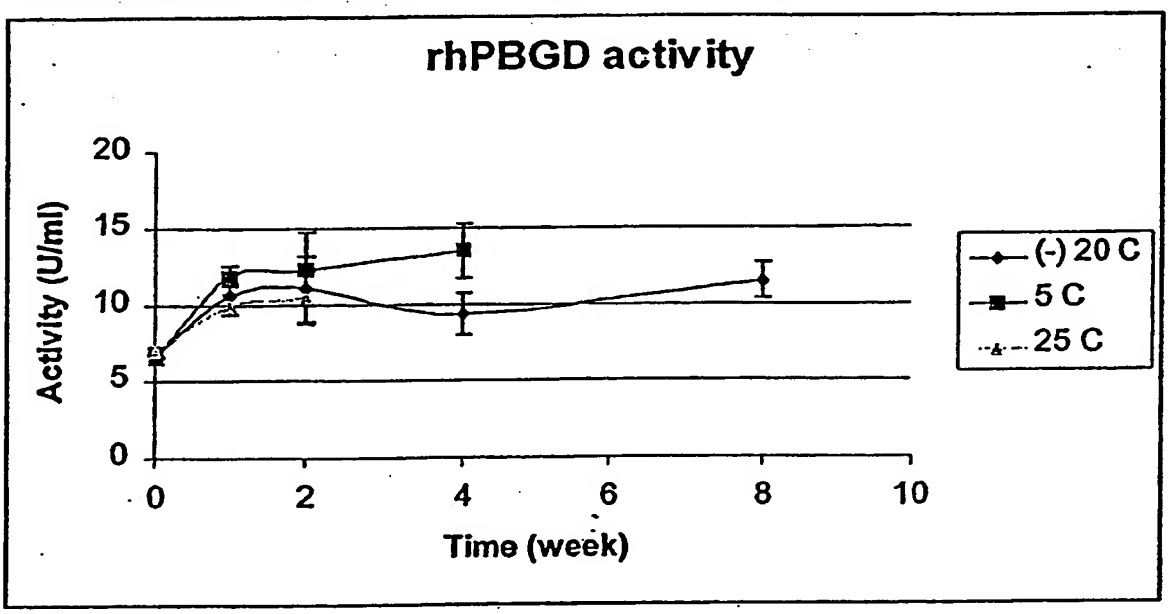

Figure 51. The rhPBGD activity measured over 8 weeks. The stability study has been performed under nitrogen at –20°C ± 5°C, 5°C ± 3°C and at 25°C ± 2°C.

Fig. 48

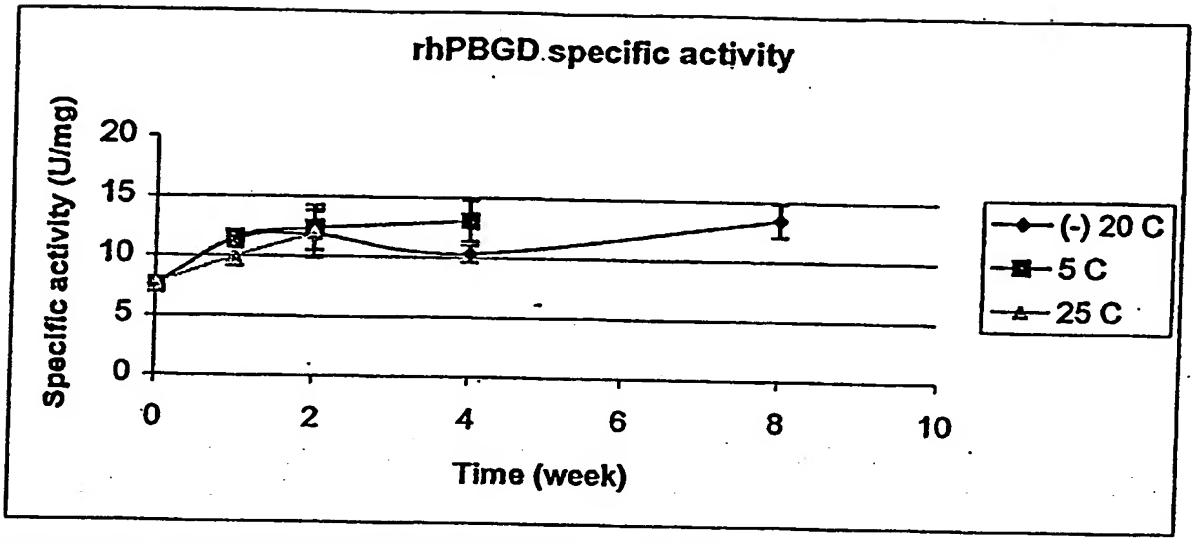

Figure 52. The specific rhPBGD activity measured using the enzyme activity assay and BCA protein concentration assay. The stability study has been performed under nitrogen at –20°C ± 5°C, 5°C ± 3°C and at 25°C ± 2°C.

Fig. 49